Reference: *Biol. Bull.* **187:** 48–54. (August, 1994)

Glutamate Immunoreactivity in Non-neuronal Cells of the Sea Anemone *Metridium senile*

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Abstract. The distribution of glutamate in the tentacles and oral disk of the sea anemone *Metridium senile* was investigated by wholemount immunohistochemistry with the use of a monoclonal antibody raised against the derivatization product γ-L-glutamyl-L-glutamic acid. Immunoreactivity was localized in one class of tentacle nematocysts and on their associated threads. These nematocysts were concentrated at the distal end of tentacles, none being found at the base of tentacles or in the oral disk. Muscle end-feet of epitheliomuscular cells also stained in the longitudinal muscle of tentacle ectoderm. In contrast, immunostaining in the oral disk was confined to ectodermal granule-containing cells overlying the radial muscle. These results support a role for glutamate as an osmolyte precursor in nematocysts but provide little clue as to the functional significance of this amino acid in muscle and gland cells.

Introduction

Glutamate has been identified as an excitatory neurotransmitter in the vertebrate central nervous system (CNS) (Fonnum, 1984; Monaghan *et al.*, 1989) as well as in invertebrates (Duce, 1988; Leake and Walker, 1980). It is established as the excitatory neuromuscular transmitter in arthropods (Usherwood, 1980). Among lower invertebrates, glutamate has been localized by immunohistochemistry in a small population of leech CNS neurons that may be associated with feeding behavior as well as with initiation of swimming (Brodfuehrer and Cohen, 1990, 1992). Glutamate also appears to act as an excitatory neuromuscular transmitter in the flatworm *Hymenolepis diminuta*, and glutamate immunoreactivity has been detected in the longitudinal nerve cord of this species (Webb, 1988; Webb and Eklove, 1989). Thus glutamate appears to be broadly distributed and evolutionarily conserved in the nervous systems of invertebrates.

Because cnidarians are considered to be modern representatives of the earliest organisms known to possess a nervous system (Bullock and Horridge, 1965; Anderson, 1990; Mackie, 1990), it is of interest to know whether amino acid transmitters such as glutamate play a neurotransmitter role in this group. Neuropeptides of the FMRFamide family were identified and localized to neurons in species from all three cnidarian classes: Hydrozoa, Scyphozoa, and Anthozoa (Grimmelikhuijzen *et al.*, 1992, for review; Anderson *et al.*, 1992). Similarly, conventional transmitters such as monoamines were detected in various cnidarians (Carlberg and Rosengren, 1985; De Waele *et al.*, 1987; Chung *et al.*, 1989; Pani and Anctil, 1994), and monoamine-containing neurons were visualized by immunohistochemistry in the anthozoan *Renilla koellikeri* (Umbriaco *et al.*, 1990; Anctil, 1990) and the hydrozoan *Hydra* (Carlberg, 1992; Carlberg and Anctil, 1993, for review). Amino acids, in contrast, have attracted less attention, except recently when taurine was reported to act electrophysiologically as a neurotransmitter on, and to be present within neurons of, the ectodermal motor nerve-net of the jellyfish *Cyanea capillata* (Anderson and Trapido-Rosenthal, 1990; Carlberg *et al.*, 1993).

Carlyle (1974) reported that glutamic acid reversibly depressed electrically stimulated contractions of oral margin/sphincter muscle preparations in the sea anemone *Actinia equina*, an effect shown to be pharmacologically specific. McFarlane *et al.* (1990) confirmed this glutamate response on sphincter muscle preparations of the sea anemones *Calliactis parasitica* and *Tealia felina*. Furthermore, Carlyle (1974) produced biochemical evidence for the presence of large amounts of releasable glutamic acid

Received 28 February 1994; accepted 25 May 1994.
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in the oral margin of *A. equina*. Taken together, these observations suggest a possible transmitter role for glutamic acid in sea anemones.

An important criterion for validating glutamate as a neurotransmitter substance requires that glutamate be localized within neurons. The present immunocytochemical study was undertaken in an attempt to provide such evidence in the sea anemone *Metridium senile* and to search for a cellular substrate for the above-mentioned modulatory action of glutamate. Using a monoclonal antibody raised against a derivatized glutamate dipeptide, we visualized immunoreactive ectodermal cells, but not neuronal cells, in the tentacles and oral disk of this species.

Materials and Methods

Specimens of the sea anemone *Metridium senile* were obtained from Pacific Bio-Marine Laboratories (Venice, CA) and Marinus Inc. (Long Beach, CA). They were maintained for several months in recirculated and filtered artificial seawater (ASW) at 12–14°C. The animals were starved for at least 2 days before they were anesthetized by gradually replacing the seawater in which they were immersed with a 1:1 mixture of 0.37 *M* $MgCl_2$ and ASW. Several body regions were excised, although the tentacles and oral disk were investigated in the greatest detail.

The immunocytochemical procedure is based on the use of a monoclonal antibody characterized by Madl *et al.* (1986). The antibody, purchased from INCSTAR, was raised in mouse against the dipeptide γ-L-glutamyl-L-glutamic acid (γ-Glu-Glu) conjugated to keyhole limpet hemocyanin *via* glutaraldehyde-borohydride. To convert endogenous glutamate to γ-Glu-Glu, tissues were first immersed in 5% carbodiimide [(1-ethyl-3-dimethylaminopropyl)carbodiimide] (Sigma) freshly made in 0.1 *M* phosphate-buffered saline (PBS, adjusted with NaCl to seawater osmolarity) for 10–25 min at pH 7.2 and 4°C. This was immediately followed by a fixation with 4% glutaraldehyde in PBS for 80 min at pH 7.2 and 4°C. Tissues were rinsed three times in PBS under these conditions over a period of 12–48 h.

Most of the tentacle and oral disk tissues were then processed directly for wholemount immunocytochemistry. Some column and pedal tissue blocks were immersed in a graded series of sucrose (up to 30%) in PBS and embedded in O.C.T. compound (Miles Laboratories). The latter were frozen by immersion in isopentane chilled with dry ice. For tissue sectioning, 15-μm sections were prepared using a Hacker-Bright cryotome and deposited on gelatin-chromalum-coated sections.

For immunocytochemistry of either wholemount preparations or sections, tissues were first incubated in 0.1% H_2O_2 for 10 min to remove endogenous peroxidases, then blocked in 10% normal goat serum diluted in PBS-TX (0.3% Triton-X-100 in PBS) for 20 min. After tissues were rinsed in PBS (3 × 5 min), they were incubated in the primary antibody diluted 1:750 in PBS-TX for 16–24 h at 4°C. After rinsing as above, tissues were incubated for 12–24 h at 4°C in a goat anti-mouse, peroxidase-labeled secondary antibody, diluted 1:100 in PBS-TX. Tissues were rinsed (3 × 5 min) in 0.9% NaCl in 0.05 *M* Tris at pH 7.6, then reacted with 0.5% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.01% H_2O_2 in NaCl/Tris buffer for 10–25 min. Sections were mounted in Aquamount (BDH) and wholemounts in glycerol:PBS (3:1).

As a control procedure for the immunostaining, the primary or secondary antibody was omitted on a few tentacle and oral disk preparations from all 13 animals processed in this study. In addition, some wholemounts from four animals were incubated in the primary antibody (diluted 1:750) preadsorbed overnight at 4°C with 1 mg/ml each of γ-Glu-Glu, *N*-acetyl-L-glutamic acid (*N*-Ac-Glu), L-aspartyl-L-glutamic acid (Asp-Glu) or L-glutamic acid (all from Sigma), after centrifugation for 5 min at 14,000 × *g* and retention of the supernatant.

Results

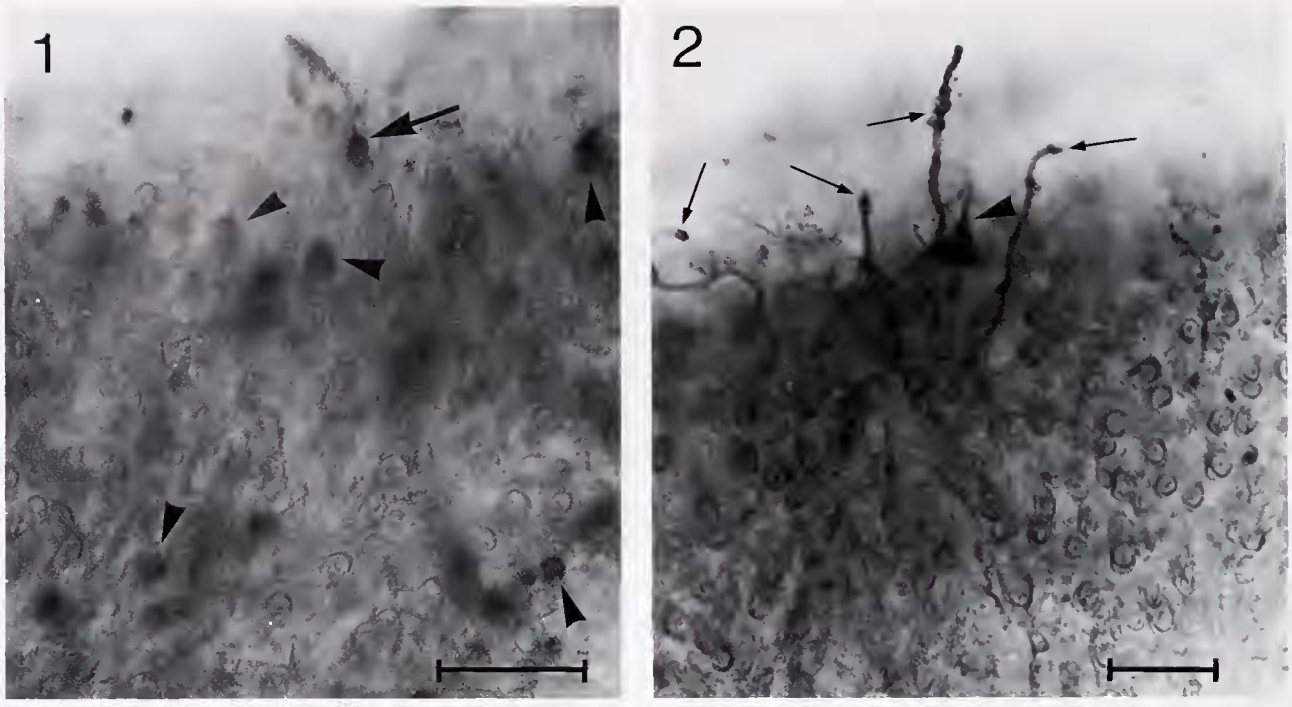
Detection of glutamate immunoreactivity

The following observations are based on wholemount preparations from 11 animals as immunostaining in sections was less well preserved or added no information beyond that available in wholemounts. Three types of immunoreactive elements were detected: ectodermal nematocysts in tentacles (Figs. 1–4), ectodermal muscle cells in tentacles (Fig. 5), and ectodermal granule-containing cells of the oral disk (Figs. 6–7). Only the last type was not detected in all animals examined, being present in oral disk preparations of six animals. No immunostaining was detected in the column and pedal disk of the specimens.

There was no detectable staining in preparations in which the primary or secondary antibody was omitted from the protocol or the primary antibody was preadsorbed with γ-Glu-Glu (Fig. 4). Preparations fixed with glutaraldehyde without prior treatment with carbodiimide also failed to stain. Preadsorption of antibody with *N*-Ac-Glu or Asp-Glu resulted in tentacles with a staining pattern similar to that obtained with untreated antibody, except that the maximum nematocyst staining as seen in Figure 1 was never observed. Preadsorption of antibody with glutamic acid resulted in a staining reaction that was indistinguishable from that associated with untreated antibody.

Nematocyst immunostaining

The most readily observed immunostaining was associated with tentacle nematocysts and their released fila-



Figures 1, 2. Glutamate immunoreactive elements of tentacle nematocysts (wholemounts). Scale bars = 25 μm.

Figure 1. Stained nematocyst capsules. Note several stained, noneverted capsules (arrowheads) as well as capsule with everted thread that is heavily stained at its apex (arrow). Note also that most of the nematocyst capsules did not stain.

Figure 2. Stained everted threads showing tapering shaft (arrowhead) and decorated threads (arrows) reflecting their coiled or twisted state and the presence of spines.

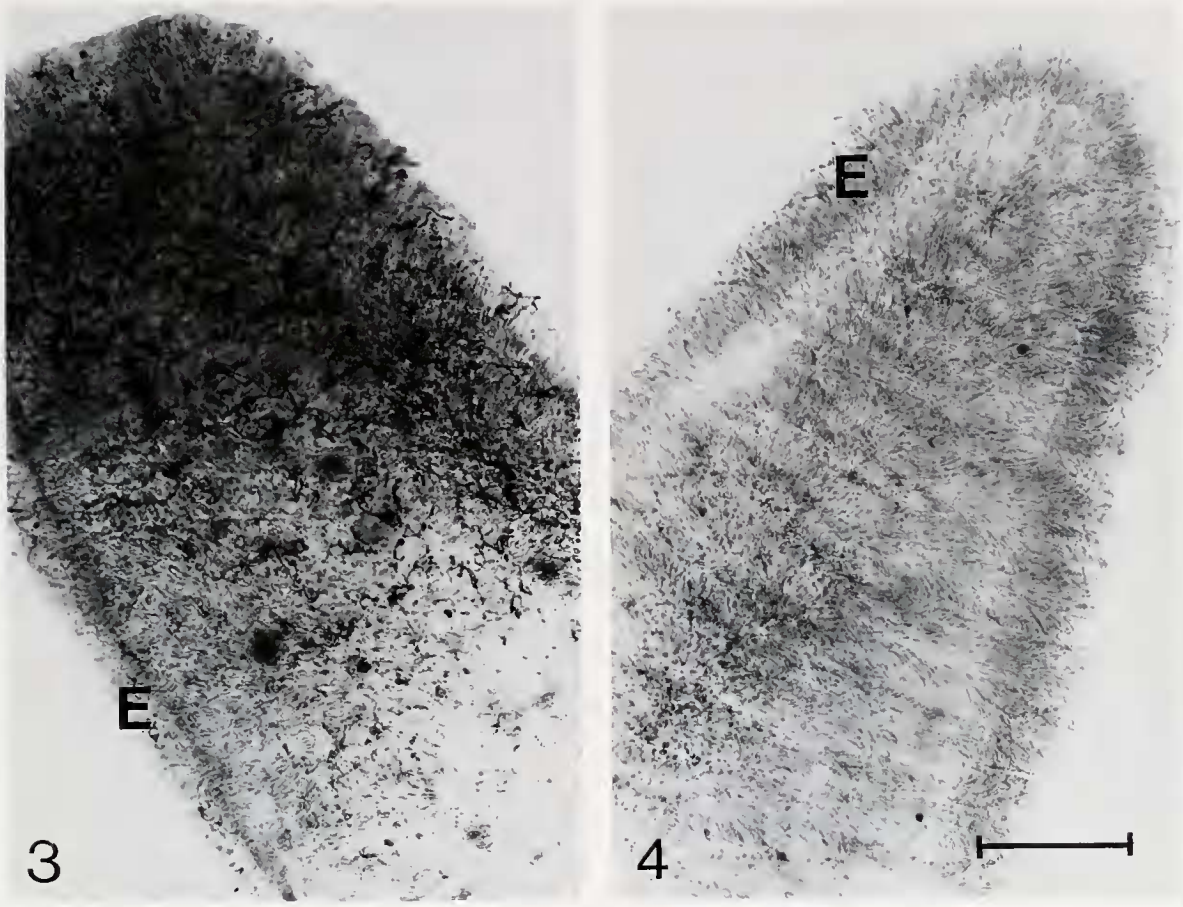
ments (Figs. 1, 2). Nematocyst immunoreactivity was invariably concentrated distally on the tentacles on both their oral and aboral sides. Although all animals tested with the primary antibody displayed positive immunoreactive responses, the intensity of staining and number of stained elements varied substantially, even between tentacles from the same animal. From the tip of the tentacles, there was a more or less gradual decline in the number and intensity of the immunostained elements, until little or no staining was detected in the proximal two-thirds or half of the tentacle (Fig. 3).

Only a relatively small proportion of discharged or undischarged nematocysts present in the stained areas were immunoreactive, and in those nematocysts staining was mainly associated with the capsule wall (Fig. 1). There was an ascending gradient of staining intensity from the base to the distal end of the capsule where the shaft is anchored (Fig. 1). The nematocytes in which these capsules lie showed no staining reaction. The immunoreactive capsules seemed to belong to a single morphological type measuring 15–20 μm by 3–4 μm. This corresponds roughly to the microbasic b-mastigophore of ordinary tentacles as described in this species by Westfall (1965).

Many of the immunoreactive capsules displayed everted shafts and threads that stained also (Fig. 2). When these elements were completely uncoiled, reaching a length of up to 80 μm, it was possible to observe a shaft, 2 μm in diameter, emerging from the capsule and gradually tapering to a thread of 1 μm in diameter. This is also diagnostic of the microbasic b-mastigophore (Westfall, 1965; Mariscal, 1974). The strong staining reaction at the tip of some ordinary tentacles (Fig. 3) was in fact due to a meshwork of such immunoreactive shafts and threads that covers the surface of the ectoderm. These discharged filaments appear variously decorated, reflecting the extent to which they are coiled, folded, or endowed with spines. Staining seemed to be localized on the surface of the filaments (Fig. 2). Reduction of both stained nematocyst capsules and stained discharged filaments accounts for the lack of immunostaining in the proximal part of the tentacles.

Ectodermal muscle cell immunostaining

This staining reaction appears as a more or less continuous layer of processes, 2–4 μm in depth, at the base



Figures 3, 4. Glutamate immunoreactivity in nematocysts of a tentacle (Fig. 3) and its absence in another (control) tentacle (Fig. 4) from the same specimen of *Metridium senile* (wholemounts). E, ectoderm.
Figure 3. Density of stained nematocysts diminishes from distal tip toward the base of the tentacle.
Figure 4. Tentacle was treated with primary antibody preabsorbed with γ-Glu-Glu; no staining was detected. Scale bar = 100 μm.

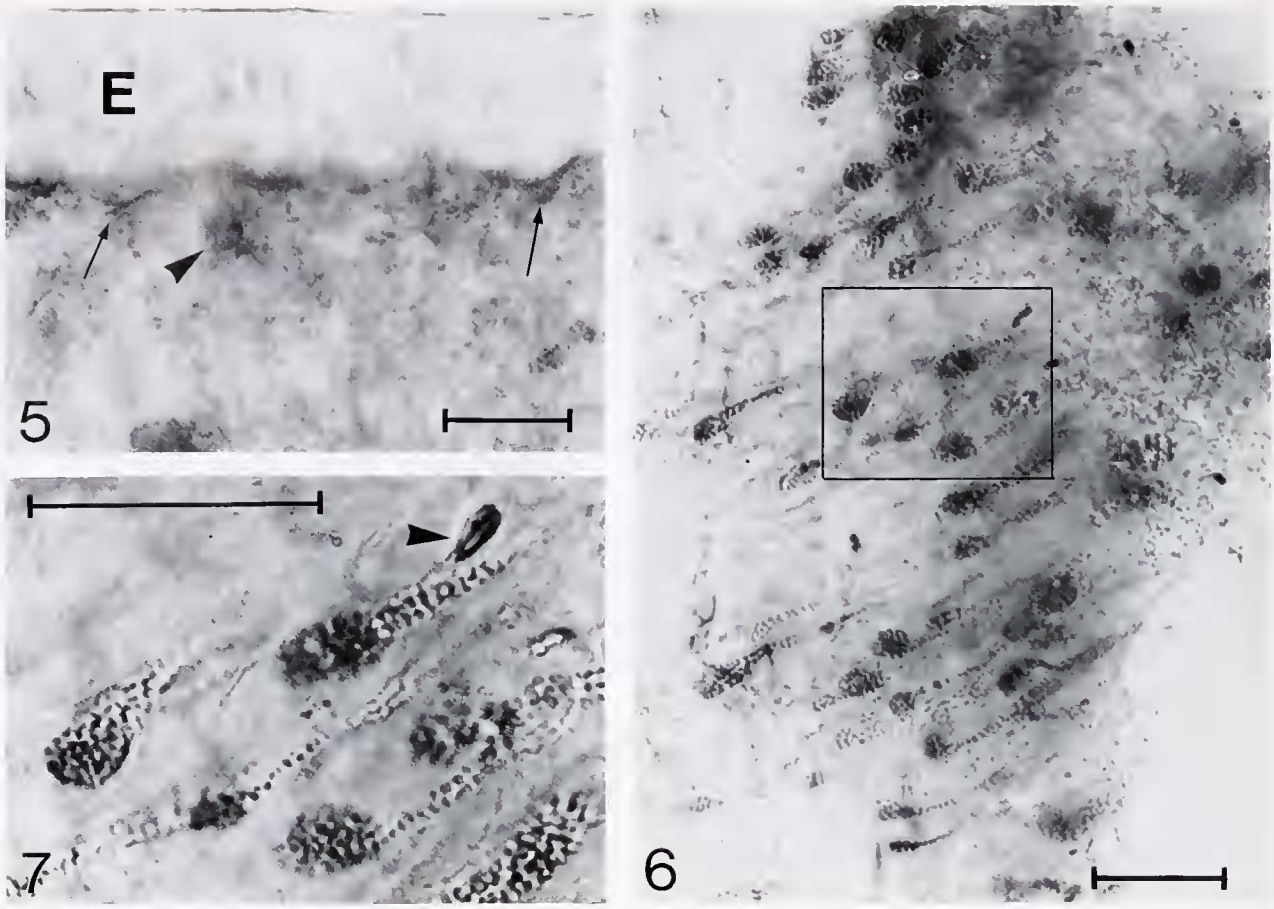
of the ectoderm (Fig. 5). Many such processes have a twisted or folded appearance (Fig. 5), presumably as a result of buckling in the tentacle. They seem to correspond to the layer of muscle feet of the musculoepithelial cells of the longitudinal musculature, associated cell bodies showing no clear evidence of staining (Fig. 5). Staining was present on both oral and aboral sides of the tentacles and usually extended from the base of the tentacles to within a short distance (0.1–0.3 mm) of their tip.

Ectodermal granule-containing cells

The third immunoreactive site was localized in the ectoderm of the oral disk proximal to the base of the tentacles (Fig. 6). The immunoreactivity was associated with pear-shaped cells (9 μm in perikaryon length) tapering to a single axon-like process and filled with large granules, 1–2 μm in diameter (Figs. 6, 7). There was no clear evidence that the granules themselves had stained. The processes were up to 40 μm in length and approximately parallel. The stained granular cells were distributed over the radial muscle in that area. The radial muscle itself was not stained.

Discussion

Our observations demonstrate the presence of glutamate immunoreactivity in the tentacles and oral disk of *M. senile*, in agreement with Carlyle's (1974) biochemical detection of large amounts of glutamic acid in the oral margin of the sea anemone *A. equina*. The lack of specificity of induced glutamate release from oral margin/sphincter preparations stopped Carlyle (1974) short of proposing a neurotransmitter role for this amino acid in this species; instead he proposed that "nematocysts and mucus-secreting cells may be potential sources of release" (Carlyle, 1974). Our results give credence to the latter view, because glutamate immunostaining was associated



Figures 5–7. Glutamate immunoreactive cells in the ectoderm of tentacles and in the oral disk (whole-mounts). Scale bars = 25 μm.

Figure 5. Layer of stained muscle feet of longitudinal muscle at the base of the ectoderm (E) in tentacle. Note out-of-focus cell body of musculoepithelial cell (arrowhead) as well as evidence of buckling (arrows). The apparent position of the cell body below the ectoderm is an artifact caused by tilting at the edge of the tentacle.

Figures 6–7. Low-power (Fig. 6) and high-power (Fig. 7) views of a field of granule-containing cells in the oral disk. Rectangle in Figure 6 delimits enlarged area shown in Figure 7. The dark background in Figure 6 represents the position of underlying radial muscle fields. Note parallel orientation of cell processes. Note also in Figure 7 that the granular texture of these cells is apparent in both cell bodies and processes. Arrowhead in Figure 7 indicates a nematocyst.

only with non-neuronal cells of *M. senile*, among which nematocysts and granular cells figure prominently.

Specificity of the immunostaining

Control procedures indicated that the observed immunostaining represents specific antibody binding to γ-Glu-Glu. The absence of effect or small effect of preadsorption tests with glutamate, *N*-Ac-Glu, and Asp-Glu on immunostaining in our preparations is largely consistent with the results of corresponding tests elsewhere (Madl *et al.*, 1986). Moreover, the absence of immunostaining in preparations fixed without carbodiimide all but excludes the presence of endogenous γ-Glu-Glu in the tissues.

Nematocyst immunostaining

Reports of large amounts of polymeric γ-glutamic acids in cnidarian nematocysts, including sea anemones (Weber, 1990, 1991), may appear to account for the nematocyst staining described in this study. However, such is apparently not the case, because omission of the carbodiimide treatment eliminated nematocyst immunostaining. In addition, staining was intense only in the periphery of the distal part of the capsule interior, not in the core (matrix) of the nematocysts where the polymers are expected to be concentrated (Weber, 1991). Thus nematocyst immunoreactivity in *M. senile* may represent a pool of free glutamate either recruited in the

polymerization process or resulting from enzymatic hydrolysis of already-formed polymers (Weber, 1994). This immunostaining lends some indirect support to Weber's hypothesis that such polymers act as substrate for generating high osmotic pressures inside the nematocyst capsule, thus providing an intrinsic force for nematocyst discharge (Weber, 1989, 1991).

The question arises as to why only one category of nematocysts displayed immunostaining when polymeric γ-glutamic acids were reported also in other categories of nematocysts (Weber, 1991). One possibility is that other nematocyst classes may contain less of these polymers, therefore less free glutamate, than the microbasic b-mastigophores identified in this study. If these nematocysts concentrate such polymers more than the other kinds do, then one may expect that they discharge their thread more readily, owing to a greater buildup of osmotic pressure, than do the other kinds. This appears to be the case, because microbasic b-mastigophores are known to be easily discharged in *Metridium* (Westfall, 1965).

Our results show that the released shaft and thread of these nematocysts were stained, but that staining seemed to be associated only with their external surface. Such a staining pattern may have resulted from adhesion of the immunoreactive material in the nematocyst capsule on the filaments. A means of fortuitous non-neuronal release of glutamate—of the kind suggested by Carlyle (1974)—is thus provided, and one must use particular caution in interpreting studies of amino acid release in such animals. Whether the putative glutamate sticking on the filaments has any role associated with nematocyst discharge remains to be explored. Another unresolved matter is whether the uneven spatial distribution of stained nematocysts on tentacles represents an intrinsic arrangement of microbasic b-mastigophores conferring some functional specificity or reflects merely the skewed distribution of a subpopulation of these nematocysts.

Ectodermal cell immunostaining

Glutamate immunostaining in ectodermal muscle and granular cells is an unexpected finding. To our knowledge this is the first time that a putative neuroactive substance has been immunohistochemically localized in this type of muscle cell. In contrast, ectodermal gland cells of tentacles of the jellyfish *Cyanea lamarcki* were reported to show serotonin immunoreactivity (Elofsson and Carlberg, 1989), and dopamine-immunoreactive granule-containing cells strikingly similar in morphology to those of the present study were described by Carlberg (1992) in tentacles and peristomium of *Hydra attenuata*. The latter and the granular cells identified in our study are not epidermal mucous cells because their processes are directed inward, not toward the external surface as in mucous cells. Cells with similar morphology, size, and granule content were demonstrated by scanning electron microscopy in oral tentacles of the cerianthid *Cerianthcopsis americanus* ("gland cells" of Fig. 9 in Fautin and Mariscal, 1991). Thus these may be secretory cells, with glutamate as a putative secretory product serving some paracrine role. Because the distribution of these cells coincides with that of radial muscle fields in the oral disk, it is possible that this muscle layer constitutes one of the functional targets of the cell secretions.

Glutamate has been identified as a feeding stimulant in the sea anemones *Actinia equina* (Steiner, 1957) and *Calliactis parasitica* (McFarlane, 1975) by eliciting mouth opening. Combined with its relaxing effect on the sphincter muscle (Carlyle, 1974), a prerequisite for mouth opening (McFarlane, 1970), these responses would suggest that glutamate is involved in the coordination of feeding in sea anemones, although our study failed to find evidence of glutamate-immunoreactive cells in the vicinity of the sphincter muscle itself. Other prefeeding responses include expansion of the radial muscle in the oral disk and extension of the tentacles, both associated with activity in an ectodermal slow conducting system, possibly a nerve-net (McFarlane, 1970; Lawn, 1975; McFarlane *et al.*, 1993). Although ectodermal muscle feet in *Metridium* tentacles apparently contain free glutamate, it is not clear how this glutamate is involved in these feeding responses.

In this study we have provided immunohistochemical evidence that, in the sea anemone *Metridium*, the amino acid glutamate or a related substance is stored only in non-neuronal cells; in contrast, in the scyphozoan jellyfish *Cyanea*, similar evidence suggests that another amino acid, taurine, is present only in neurons (Carlberg *et al.*, 1993). This disparity suggests that profoundly different paths of cellular commitment for processing amino acid transmitters may be expressed among cnidarian species.

Acknowledgments

This study was funded by grant OGP0006447 from the Natural Sciences and Engineering Research Council of Canada to M.A.

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